

IN THE CLAIMS

Claim 1 (original): The method of non-enzymatic harvesting and in vitro culturing corneal epithelial cells for transplantation comprising the steps of: a) dissecting corneal endothelial cells from a tissue source; growing said corneal endothelial cells at a low density in extracellular matrix coated (ECM) culture plates for a period of time; b) passaging said cells into a secondary culture system wherein the secondary culture system is comprised of ECM coated culture plates and the addition of sufficient cellular growth factors; c) growing the corneal endothelial cells to confluency; and d) harvesting the corneal endothelial cells from the second culture system in sufficient quantities to be useful in transplantation to a subject in vivo.

Claim 2 (original): The method of claim 1 wherein the dissection of the corneal endothelial cells further comprises the step of removing the cells from the tissue source such that the cells are dissected away from the stroma of said tissue source prior to growing in the first culture system.

Claim 3 (original): The method of claim 2 wherein said tissue source can be corneal buttons or rims.

Claim 4 (original): The method of claim 2 wherein the ECM is comprised of Bovine corneal endothelial cell extracellular matrix (BCE-ECM).

Claim 5 (original): The method of claim 2 wherein the ECM is comprised of an artificially generated extracellular matrix (AG-ECM).

Claim 6 (original): The method of making ECM coated plates comprising the steps of: a) seeding bovine corneal endothelial cells (BCEC) onto dishes in DME-H16 medium containing

approximately 10% Fetal Calf Serum, 5% Calf Serum, 5% Dextran, 300 µg/ml glutamine, 2.5 µg/ml Amphotericin B, and 50 ng/ml bFGF; b) growing BCEC until confluent; c) treating the dishes will be treated with NH₄OH at a volume sufficient to cover at least 2/3 of the plate for at least about 5 minutes whereafter the NH₄OH removed; and d) storing the BCEC coated at 4 °C about a week prior to use in order to eliminate any surviving BCEC.

Claim 7 (original): The method of claim 5 wherein the artificially generated endothelial cell extracellular matrix (AG-ECM) is made by the process comprising: a) fibronectin, laminin and RGDS (Arg-Gly-Asp-Ser peptide) are prepared in a 100 µg/mL in distilled water; b) collagen type IV is prepared at a concentration of about 1 mg/mL in 0.01% acetic acid ; c) basic fibroblast growth factor (bFGF) is prepared at a concentration of about 100 µg/mL in bovine serum albumin (0.05% w/v); d) the solutions of steps a, b, and c are mixed and then incubated at 4 °C for two hours; and e) the mixture of step d is diluted about 1:10 with phosphate buffered saline, and then a sufficient amount of the solution is added to a dish and allowed to stand at 4 °C for approximately 1 hour before use.

Claim 8 (original): Human corneal endothelial cells (HCEC) suitable for use in transplantation made using the method of claim 1.

Claim 9 (original): Human corneal endothelial cells (HCEC) suitable for use in transplantation made using the method of claim 2.

Claim 10 (original): Human corneal endothelial cells (HCEC) suitable for use in transplantation made using the method of claim 5.

Claim 11 (original): An apparatus for growing cells in culture having at least one surface which is in contact with the cells

and wherein the surface is coated with a mixture comprising BCE-ECM prior to use.

Claim 12 (original): The apparatus of claim 11 selected from the group consisting of: cell culture plates and flasks.

Claim 13 (original): The apparatus of claim 11 wherein the cells are mammalian cells.

Claim 14 (original): An apparatus for growing cells in culture having at least one surface which is in contact with the cells and wherein the surface is coated with a mixture comprising AG-ECM prior to use.

Claim 15 (original): The apparatus of claim 14 wherein the cells are mammalian cells.

Claim 16 (original): A method of making HCEC cells wherein said HCEC are lacking class I HLA antigens comprising the steps of: a) dissecting human corneal epithelial cells from a neonatal source such that said cells do not express class I HLA antigens; b) growing said corneal epithelial cells at a low density in a range of about 100 to 500 cells per square millimeter in a primary culture system comprising extracellular matrix coated (ECM) culture plates for a period of time; c) passaging said cells into a secondary culture system wherein the secondary culture system is comprised of an ECM coated culture plates and the addition of sufficient cellular growth factors; d) growing said cells until the cells are confluent; and e) harvesting said cells from the second culture system in sufficient quantities to be useful in transplantation to a subject in vivo.

Claim 17 (original): The method of claim 16 wherein the ECM is comprised of Bovine corneal endothelial cell extracellular matrix (BCE-ECM).

Claim 18 (original): The method of claim 16 wherein the ECM is comprised of an artificially generated extracellular matrix (AG-ECM).

Claim 19 (original): Human corneal endothelial cells (HCEC) suitable for use in transplantation made using the method of claim 16.

Claim 20 (original): A method of making HCEC cells wherein said HCEC are lacking class I HLA antigens comprising the steps of: a) dissecting human corneal epithelial cells from a tissue source, growing said corneal epithelial cells at a low density in a range of about 100 to 500 cells per square millimeter in a primary culture system comprising extracellular matrix coated (ECM) culture plates for a period of time; b) passaging said cells into a secondary culture system wherein the secondary culture system is comprised of an ECM coated culture plates and the addition of sufficient cellular growth factors; c) growing said cells to confluency; d) harvesting said cells from the second culture system in sufficient quantities to be useful in transplantation to a subject in vivo; and e) transforming said cells such that the a cell line is created and said cells contain a targeted disruption in the HLA gene locus thereby inhibiting expression of HLA antigens.

Claim 21 (original): Human corneal endothelial cells (HCEC) suitable for use in transplantation made using the method of claim 20.

Claim 22 (original): The method of claim 1, wherein the genotype of each HCEC cell line is determined using gel-based detection methods, using non-gel-based detection methods or with genetic markers.

Claim 23 (original): The method of claim 1, wherein the target immunotype of each HCEC cell line is determined using serological or molecular methods.

Claim 24 (original): The method of claim 20, wherein the target immunotype is determined by HLA tissue typing.

Claim 25 (original): A cell depository comprising multiple populations of HLA-typed HCEC cell lines, wherein each HCEC cell line is derived from a different donor and is homozygous for a unique HLA haplotype.

Claim 26 (original): The cell depository of claim 25, wherein the HCEC cell lines are obtained from donors of different ethnicities.

Claim 27 (original): The cell depository of claim 25, wherein the contents of the depository are catalogued.

Claim 28 (original): A method for producing an HCEC cell depository of genotyped HCEC cells from multiple donors comprising the steps of: (a) selecting donors; (b) determining the genotype of each donor; (c) isolating HCEC cells from primary cultures obtained from each donor; (d) culturing the isolated HCEC cells to obtain HCEC cell lines; (e) determining the genotype of each HCEC cell line; and (f) cataloging the genotype of each HCEC cell line obtained in (g).

Claim 29 (original): A method for producing a HCEC cell depository of immunotyped HCEC cells from multiple donors comprising the steps of: (a) selecting donors; (b) determining the immunotype of each donor; (c) developing primary cultures of HCEC cells; (d) isolating HCEC cells from each donor; (e) culturing the isolated HCEC cells to obtain HCEC cell lines; (f) determining the immunotype of each HCEC cell line; and (g) cataloging the immunotype of each HCEC cell line obtained in (e).

Claim 30 (original): A method for producing a HCEC cell depository of genotyped and immunotyped HCEC cells from multiple donors comprising the steps of: (a) selecting donors; (b) determining the genotype and immunotype of each donor; (c) developing primary cultures of HCEC cells; (d) isolating HCEC cells from each donor; (e) culturing the HCEC cells to obtain HCEC cell lines; (f) determining the genotype and immunotype of each HCEC cell line; and (g) cataloging the genotype and immunotype of each HCEC cell line obtained in (e).

Claim 31 (original): The method of claim 28, wherein the donors are mammalian.

Claim 32 (original): The method of claim 28, wherein the donors are human.

Claim 33 (original): The method of claim 29, wherein the donors are human.

Claim 34 (original): A method of transporting HCEC for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC into a flask or suitable container filled with culture medium; and c) transporting said membrane coated with the HCEC.

Claim 35 (original): The method of claim 34 wherein said target tissue is a corneal button.

Claim 36 (original): The method of claim 34 wherein said target tissue is a secondary culture system.

Claim 37 (original): A method of transporting HCEC for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor

tissue for a time period sufficient to prevent dislodging during transportation; and d) transporting said tissue in a storage medium.

Claim 38 (original): The method of claim 37 wherein the target tissue is a denuded cornea.

Claim 39 (original): The method of claim 34 wherein the biodegradable polymer comprises a semi-solid state suitable for coating with BCE-ECM or other biocompatible coating such as Diamond-Like-Carbon.

Claim 40 (currently amended): A method for protecting the regenerated corneal button ~~target tissue of claim 35~~ from denuding during transport or implantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor tissue for a time period sufficient to prevent dislodging during transportation in the presence of 1% sodium hyaluronate which has been conjugated to bFGF; and d) transporting said tissue in a storage medium having 1% sodium hyaluronate conjugated to bFGF.

Claim 41 (original): The method of claim 40 wherein the target tissue is a denuded cornea.

Claim 42 (original): The target tissue treated according to claim 40.

Claim 43 (original): A method of storing HCEC regenerated target tissue for transplantation comprising the steps of: a) growing HCEC according to the method of claim 2 upon a biodegradable polymer membrane to confluency; b) placing the membrane coated with the HCEC onto a donor target tissue; c) growing the HCEC on the donor tissue for a time period sufficient to prevent

dislodging during transportation; d) adding an anti-icing or cryoprotective agent to said tissue in a storage medium; and e) storage of the target tissue at a very low temperature.

Claim 44 (original): A method for denuding a native cornea to make it suitable for transplantation or correction comprising the steps of: a) placing a corneal button or other target tissue in a suitable holder; b) adding a sufficient quantity of denuding reagent to the holder in step a so that it completely covers the target tissue; c) incubating the tissue with the denuding reagent for a sufficient period of time at approximately room temperature; and d) washing the target tissue with an appropriate buffer approximately 10 times.

Claim 45 (original): The method of claim 44 wherein the denuding reagent is comprised of a solution of Triton X at a concentration of about 0.01 to 1% v/v in phosphate buffered saline.

Claim 46 (original): The method of claim 44 wherein the incubation time is about 5 minutes.

Claim 47 (original): The method of claim 44 wherein the denuding reagent is comprised of a solution of ammonium hydroxide at a concentration of about 20 mM.

Claim 48 (original): The method of claim 47 wherein the incubation time is between about 2 to 5 minutes.

Claim 49 (original): A method for denuding a native cornea to make it suitable for transplantation or correction comprising the steps of: a) placing a corneal button or other target tissue in a suitable holder ; b) adding a sufficient quantity of distilled water to the holder in step a so that it completely covers the target tissue; c) incubating the tissue with the denuding reagent for about 15 minutes at approximately room temperature; d) aspirating off about half the volume of water; e) sweeping the

wetted endothelium mechanically from the corneal button; and f) washing the corneal button approximately 3 times with phosphate buffered saline.

Claim 50 (original): A reconstituted extracellular matrix preparation comprising: a sufficient amount of growth factor mixture and a sufficient amount of adhesion factor mixture.

Claim 51 (original): The growth factor mixture of claim 50 comprising a sufficient quantity of bFGF, EGF and polycarbophyll in a suitable biological buffer.

Claim 52 (original): The growth factor mixture of claim 51 wherein the concentrations of bFGF, EGF and polycarbophyll are approximately 3.33 pg/mL, 33.33 pg/mL and 0.33 mg/mL respectively.

Claim 53 (original): The adhesion factor mixture of claim 50 comprising a sufficient quantity of laminin, fibronectin, RGDS, and collagen IV in a suitable biological buffer.

Claim 54 (original): The adhesion factor mixture of claim 53 wherein the concentrations of laminin, fibronectin, RGDS, are approximately 83.33 ug/mL, and collagen IV is approximately 250 pg/mL.

Claim 55 (original): A method of coating a denuded cornea comprising the steps of: a) placing a corneal button or other target tissue in a suitable holder; b) washing the corneal button with phosphate buffered saline; c) adding a sufficient quantity of reconstituted extracellular matrix preparation of claim 50 to the holder in step a so that it completely covers the target tissue; d) incubating the corneal button for a sufficient period of time at approximately 4 °C; and e) washing the corneal button with phosphate buffered saline or other suitable buffer.

Claim 56 (original): The method of coating a denuded cornea according to claim 55, further comprising the step of: f) adding approximately 300 uL of 1% sodium hyaluronate to the corneal button prior to seeding of new endothelial cells.